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TI Purification and characterization of GDP-D-mannose  
4,6-dehydratase from porcine thyroid.  
AU Broschat K O; Chang S; Serif G  
SO EUROPEAN JOURNAL OF BIOCHEMISTRY, (1985 Dec 2) 153 (2) 397-401.  
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Two Chinese hamster ovary glycosylation mutants affected in the conversion  
of GDP-mannose to GDP-fucose.

AU Ripka J; Adamany A; Stanley P  
NC 3PO CA13330 (NCI)  
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SO ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, (1986 Sep) 249 (2) 533-45.  
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CY United States

**COMPLETED**

Participation of an endogenous inhibitor of fucosyltransferase  
activities in the developmental regulation of intestinal fucosylation  
processes.

AU Ruggiero-Lopez D; Biol M C; Louisot P; Martin A  
CS Department of General and Medical Biochemistry, INSERM-CNRS U. 189,  
France.  
SO BIOCHEMICAL JOURNAL, (1991 Nov 1) 279 ( Pt 3) 801-6.  
Journal code: 2984726R. ISSN: 0264-6021.

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## Mouse Lymphoma Cell Lines Resistant to Pea Lectin Are Defective in Fucose Metabolism\*

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Two mutants of the BW5147 mouse lymphoma cell line have been selected for their resistance to the toxic effects of pea lectin. These cell lines, termed PL<sup>R</sup>1.3 and PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2, have a decreased number of high affinity pea lectin-binding sites (Trowbridge, I. S., Hyman, R., Ferson, T., and Mazauskas, C. (1978) *Eur. J. Immunol.* 8, 716-723). Intact cell labeling experiments using [2-<sup>3</sup>H]mannose indicated that PL<sup>R</sup>1.3 cells have a block in the conversion of GDP-[<sup>3</sup>H]mannose to GDP-[<sup>3</sup>H]fucose whereas PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cells appear to be blocked in the transfer of fucose from GDP-[<sup>3</sup>H]fucose to glycoprotein acceptors. *In vitro* experiments with extracts of PL<sup>R</sup>1.3 cells confirmed the failure to convert GDP-mannose to GDP-fucose and indicated that the defect is in GDP-mannose 4,6-dehydratase (EC 4.2.1.47), the first enzyme in the conversion of GDP-mannose to GDP-fucose. The block in the PL<sup>R</sup>1.3 cells could be bypassed by growing the cells in the presence of fucose, demonstrating that an alternate pathway for the production of GDP-fucose presumably via fucose 1-phosphate is functional in this line. PL<sup>R</sup>1.3 cells grown in 10 mM fucose showed normal high affinity pea lectin binding.

PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cells synthesize GDP-fucose and have normal or increased levels of GDP-fucose:glycoprotein fucosyltransferase when assayed *in vitro*. The fucosyltransferases of this clone can utilize its own glycoproteins as fucose acceptors in *in vitro* assays. These findings indicate that this cell line fails to carry out the fucosyltransferase reaction *in vivo* despite the fact that it possesses the appropriate nucleotide sugar, glycoprotein acceptors, and fucosyltransferase.

The finding of decreased glycoprotein fucose in two independent isolates of pea lectin-resistant cell lines and the restoration of high affinity pea lectin binding to PL<sup>R</sup>1.3 cells following fucose feeding strongly implicates fucose as a major determinant of pea lectin binding.

Cell lines selected for resistance to toxic concentrations of lectins have helped elucidate the pathways and biological roles of glycosylation and have helped define lectin-binding

specificities (1). Previous work in our laboratories and in others has produced lectin-resistant cell lines with the following abnormalities: 1) defects in the biosynthesis of the lipid-linked oligosaccharide precursor of asparagine-linked glycoproteins (2-6); 2) a block in the processing of high mannose<sup>1</sup> species (7-9); and 3) modifications in the addition of outer sugars (*i.e.* *N*-acetylglucosamine, galactose, sialic acid, and fucose) to the processed core (8, 10-12).

In this paper we report the characterization of two pea lectin-resistant mutants of the BW5147 mouse lymphoma cell line. The mutants, PL<sup>R</sup>1.3 and PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2, were originally isolated and studied by Trowbridge *et al.* (10). The PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 line is a double mutant, produced by first isolating a BW5147 mutant resistant to kidney bean leucoagglutinin (PHA<sup>R</sup>1.8)<sup>2</sup> and then selecting for resistance to pea lectin. The PHA<sup>R</sup>1.8 cells, while having a decreased galactose and sialic acid content, have retained the parental cell line's sensitivity to pea lectin. Both pea lectin-resistant mutants show a decrease in pea lectin binding but have normal cell surface expression of three glycoprotein antigens (Thy-1, T-200, and gp69-71) (10).

We report here that the PL<sup>R</sup>1.3 and PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 lines have less glycoprotein fucose than their respective parental cell lines. In the PL<sup>R</sup>1.3 line this is due to a defect in the synthesis of the fucose donor, GDP-fucose. When this line is grown in the presence of fucose the biosynthetic defect is bypassed and the ability to bind pea lectin is restored. The defect in the PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cells appears to be at the level of fucose transfer from GDP-fucose to glycoprotein.

### EXPERIMENTAL PROCEDURES

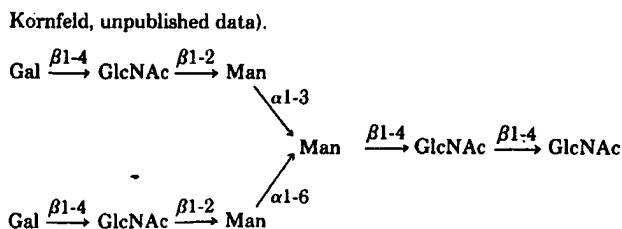
**Materials**—Materials were obtained from the following sources: D-[2-<sup>3</sup>H]mannose (18.4 Ci/mmol), L-[6-<sup>3</sup>H]fucose (11.7 Ci/mmol), GDP-D-[1-<sup>3</sup>H(N)]mannose (10.4 Ci/mmol), GDP-L-[U-<sup>14</sup>C]fucose (0.140 Ci/mmol), and Na<sup>125</sup>I, carrier free, New England Nuclear;  $\alpha$ -minimal essential medium, Flow Laboratories, Rockville, MD; fetal bovine serum (heat inactivated), penicillin, and streptomycin, Grand Island Biological Co., Triton X-100 and 3a70 scintillation cocktail, Research Products International Corp., Elk Grove Village, IL; Bio-Gel P-6 (200 to 400 mesh) Bio-Rad Laboratories; Amberlite MB-3, Chemical Dynamics Corp., South Plainfield, NJ; and pea lectin, GDP-mannose, and sugars, Sigma. Pea lectin-Sepharose was made as previously described (13). Desialized fibrinogen glycopeptide was obtained from Dr. R. Kornfeld.<sup>3</sup> Dr. O. Gabriel, Georgetown Univer-

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<sup>1</sup> Unless otherwise noted, fucose is of the L configuration while all other sugars are of the D form.

<sup>2</sup> The abbreviations used are: PHA, kidney bean leucoagglutinin; GlcNAc, *N*-acetylglucosamine; PBS, phosphate-buffered saline (0.02 M NaPO<sub>4</sub>, pH 7.4-0.14 M NaCl); PL, pea lectin.

<sup>3</sup> The original glycoprotein was obtained from Drs. J. Miletich and G. Broze of this institution. The structure of the oligosaccharide portion of the desialized Fib-1 glycopeptide is shown below (R.



oligosaccharides. After 3 days the cells were harvested, and glycopeptides were prepared by pronase digestion. An aliquot of the glycopeptide fraction was subjected to acid hydrolysis, and the released sugars were separated by paper chromatography. As shown in Table I, about 90% of the radioactivity was recovered from the parent BW5147 and the PHA<sup>R</sup>1.8 lines as [<sup>3</sup>H]mannose and the rest as [<sup>3</sup>H]fucose. In contrast, the glycopeptides of the two pea lectin-resistant lines (PL<sup>R</sup>1.3 and PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2) were strikingly deficient in [<sup>3</sup>H]fucose.

The decreased fucose content of the glycoproteins from the pea lectin-resistant lines could result from any of a number of mechanisms. These include defects in: 1) the synthesis of GDP-fucose from GDP-mannose, 2) the transport of GDP-fucose into the Golgi apparatus, and 3) the transfer of fucose to glycoprotein acceptor(s). To examine the first possibility, we analyzed the aqueous fractions of the [2-<sup>3</sup>H]mannose-labeled cells for the presence of labeled GDP-mannose and GDP-fucose. Extracts from all the cell lines contained GDP-[<sup>3</sup>H]mannose. GDP-[<sup>3</sup>H]fucose was readily detected in the extracts of the parent BW5147 and the PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cells. However, in two separate experiments, we were unable to detect any radioactive GDP-fucose in the PL<sup>R</sup>1.3 extracts (Table II).

**In Vitro Conversion of GDP-Mannose to GDP-Fucose**—The preceding experiments suggest that the defect in the PL<sup>R</sup>1.3 cells is an inability to convert GDP-mannose to GDP-fucose. Therefore, an *in vitro* assay was established to examine this conversion and to allow manipulation of the reaction conditions. Cell extracts were incubated with GDP-[<sup>3</sup>H]mannose, NAD<sup>+</sup>, and a source of NADPH, and the extent of GDP-[<sup>3</sup>H]fucose formation was determined by measuring the [<sup>3</sup>H]fucose present in mild acid hydrolysates of the incubation mixtures. In the standard assay, [<sup>3</sup>H]fucose formation was proportional to the amount of protein added, at protein concentrations above 100 µg/ml (Fig. 1). After a 5- to 10-min lag, [<sup>3</sup>H]fucose production was linear with time. While NAD<sup>+</sup> was included in the reaction mixtures, it was not required for full enzymatic activity.

In order to prove that the [<sup>3</sup>H]fucose detected by this assay

TABLE I

Radioactive sugars present in glycopeptides after *in vivo* labeling with [2-<sup>3</sup>H]mannose

The labeling of cells, preparation of the glycopeptides, and the sugar analysis were performed as described under "Experimental Procedures." Results are expressed as the percentage of total radioactivity that is present in the indicated sugar. Each assay was performed using at least 3000 cpm of glycopeptide.

Cell line	Percentage of radioactivity present in	
	Fucose	Mannose
Parent BW5147	10.3	89.7
PL <sup>R</sup> 1.3	<0.5	>99.5
PHA <sup>R</sup> 1.8	10.2	89.8
PHA <sup>R</sup> 1.8 PL <sup>R</sup> 7.2	0.96	99.0

TABLE II

Radiolabeled nucleotide sugars present in cell extracts after *in vivo* labeling with [2-<sup>3</sup>H]mannose

The labeling of cells, isolation of nucleotide sugars, mild acid hydrolysis, and identification of the released monosaccharides were performed as described under "Experimental Procedures."

Cell line	Radioactivity in	
	GDP-fucose	GDP-mannose
	cpm	
Parent BW5147	3460	1519
PL <sup>R</sup> 1.3	<25	982
PHA <sup>R</sup> 1.8 PL <sup>R</sup> 7.2	1566	3577

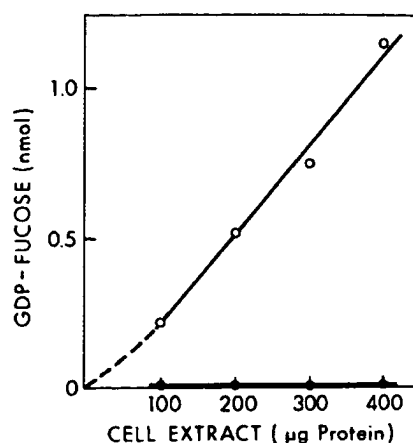


FIG. 1. Ability of cell extracts to convert GDP-[<sup>3</sup>H]mannose to GDP-[<sup>3</sup>H]fucose. Soluble cell extracts were prepared and assayed as described under "Experimental Procedures," except that the incubation time was 70 min. The soluble cell extracts contained 30 to 45% of total cell protein and greater than 90% of the GDP-mannose to GDP-fucose converting activity. ○—○, parent BW5147 extract; ●—●, PL<sup>R</sup>1.3 extract.

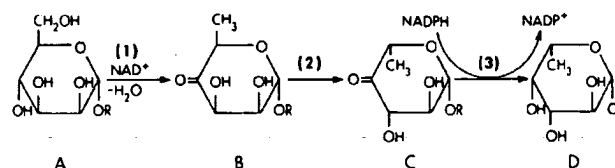


FIG. 2. Proposed pathway for the biosynthesis of GDP-fucose from GDP-mannose. From Ginsburg (23); R = GDP. Compounds are: A, GDP-α-D-mannose; B, GDP-6-deoxy-α-D-lyxo-4-hexulose (GDP-4-keto-6-deoxy-α-D-mannose); C, GDP-6-deoxy-β-L-xylo-4-hexulose (GDP-4-keto-6-deoxy-β-L-galactose); and D, GDP-6-deoxy-β-L-galactose (GDP-β-L-fucose). Enzymatic activities are: (1) 4,6-dehydratase (oxidoreductase); (2) 3,5-epimerase; and (3) 4-reductase.

is derived from GDP-[<sup>3</sup>H]fucose, we scaled up the standard incubation mixture, terminated the reaction by boiling in the absence of acid, and isolated the nucleotide sugars (see under "Experimental Procedures"). When these nucleotide sugars were hydrolyzed and the released monosaccharides isolated, [<sup>3</sup>H]fucose was found to make up 32% of the recovered radioactivity. In comparison, when the assay was performed using the standard procedure, [<sup>3</sup>H]fucose comprised 23% of the recovered label. [<sup>3</sup>H]Mannose accounted for the remainder of the radioactivity in both cases. This agreement demonstrates that the assay reflects the conversion of GDP-mannose to GDP-fucose.

The data in Fig. 1 show that PL<sup>R</sup>1.3 cell extracts lack the ability to convert GDP-mannose to GDP-fucose. Unlike the PL<sup>R</sup>1.3 line, PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cell extracts demonstrated the same ability to convert GDP-mannose to GDP-fucose as did parental cells (data not shown).

The conversion of GDP-mannose to GDP-fucose requires at least three different enzymatic activities, as shown in Fig. 2 (23-25). In a bacterial system, incubation without NADPH will cause intermediate compounds (B and possibly C in Fig. 2) to accumulate. Then, upon addition of NADPH, GDP-fucose rapidly appears without the usual time lag (23). We, therefore, performed a series of experiments to determine if the conversion of GDP-mannose to GDP-fucose in mouse cells follows the same pathway as in *Aerobacter aerogenes* and then took advantage of these results to localize more precisely the defect in the PL<sup>R</sup>1.3 line. First, we examined the ability of the parent and PL<sup>R</sup>1.3 extracts to product interme-

diates *B* and *C* from GDP-mannose. Cell extracts were incubated in reaction mixtures which lacked a source of NADPH in order to accumulate the 4-keto intermediates. At the end of the incubation time  $\text{NaBH}_4$  was added to reduce any 4-keto intermediates. The nucleotide sugars were then hydrolyzed, and the released sugars separated by paper chromatography. When compound *B* is reduced and hydrolyzed, 6-deoxy-D-talose (D-talomethylose) and 6-deoxy-D-mannose (D-rhamnose) are produced, while intermediate *C* will produce 6-deoxy-L-galactose (L-fucose) and 6-deoxy-L-glucose (23). If the 3- and 5-epimerase activities are separate enzymes, additional 6-deoxyhexoses might be detected.

The results of the trapping experiments are presented in Table III. The incubation with parental extracts yielded radioactivity that migrated with mannose, 6-deoxytalose, and rhamnose. Additional proof that the radioactivity was due to the indicated sugars was obtained by rechromatographing the individual peaks in Solvents B, D, and E. Authentic D-mannose, L-fucose, L-rhamnose, 6-deoxy-L-talose, and 6-deoxy-D-glucose were used as standards. The material labeled 6-deoxytalose in Table III cochromatographed with the standard in all three solvents. The radioactivity that chromatographed in the rhamnose area in Solvent D was predominantly rhamnose but may have contained small amounts (<10%) of 6-deoxyglucose and fucose. These data are consistent with Ginsburg's proposed pathway for the conversion of GDP-mannose to GDP-fucose (23). The data also suggest that when 4-reductase activity is inhibited, intermediate *B* accumulates to a much greater extent than *C*.

When the reductive trapping experiment was performed using PL<sup>R</sup>1.3 extracts, no 6-deoxyhexoses were detected. This finding suggests that the defect in the PL<sup>R</sup>1.3 line is in the step catalyzed by GDP-mannose 4,6-dehydratase (EC 4.2.1.47, also known as GDP-mannose 4,6-hydrolyase and GDP-mannose 4,6-oxidoreductase). The data rule out a deficient 3,5-epimerase as the sole enzyme defect in PL<sup>R</sup>1.3 cells, but it does not exclude the possibility that the cell lacks both this enzyme and the dehydratase.

To determine whether the epimerase and reductase activities are present in the PL<sup>R</sup>1.3 line, we performed the experi-

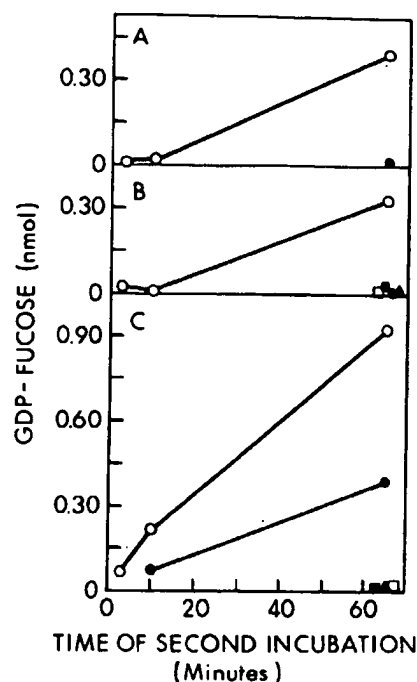


FIG. 3. Conversion of nucleotide sugar intermediates to GDP-fucose by cell extracts. GDP-[<sup>3</sup>H]mannose was preincubated with 250  $\mu\text{g}$  of soluble protein in 800- $\mu\text{l}$  final volume containing substrates and carrier as described under "Experimental Procedures," except that NADP<sup>+</sup> and glucose 6-phosphate were omitted. After 65 min of preincubation, the reaction mixtures were placed in a boiling water bath for 105 s to inactivate the extracts. The preincubations were performed as indicated. Panel B, preincubation with PL<sup>R</sup>1.3 cell extract; Panel C, preincubation with parental BW5147 extract; Panel A represents a control in which no preincubation was performed. The second incubations were initiated by the addition of 250  $\mu\text{g}$  of protein, 0.2  $\mu\text{mol}$  NAD<sup>+</sup>, 10  $\mu\text{mol}$  of nicotinamide, and 5.0  $\mu\text{mol}$  of ATP in 50 mM Tris-Cl, pH 8.0 (with or without 0.2  $\mu\text{mol}$  of NADP<sup>+</sup> and 4.0  $\mu\text{mol}$  of glucose 6-phosphate, as indicated) in a volume of 290  $\mu\text{l}$ . In the incubations without fresh cell extract, 0.3  $\mu\text{mol}$  of NADPH was added instead of NADP<sup>+</sup> and glucose 6-phosphate. The second incubations with either fresh parental ( $\circ$ ,  $\square$ ) or PL<sup>R</sup>1.3 ( $\bullet$ ,  $\blacksquare$ ) extract and either with a source of NADPH ( $\circ$ ,  $\bullet$ ) or without one ( $\square$ ,  $\blacksquare$ ) were performed for the indicated times.  $\Delta$ , reactions with NADPH but without cell extract.

TABLE III

Radioactive compounds found after incubating GDP-[<sup>3</sup>H]mannose with cell extracts in the absence of NADPH, followed by reduction and mild acid hydrolysis

GDP-[<sup>3</sup>H]mannose ( $3.68 \times 10^5$  cpm; 0.035 nmol) was incubated with 400  $\mu\text{g}$  of supernatant protein as described under "Experimental Procedures" except that exogenous NADP<sup>+</sup> and glucose 6-phosphate were omitted. After 60 min at 37°C, the reaction mixtures were heated for 105 s in a boiling water bath, and insoluble protein was removed by centrifugation at  $12,000 \times g$  for 10 min.  $\text{NaBH}_4$  (1 mg) was then added to the supernatant fluid. After 3 h at room temperature, 60  $\mu\text{l}$  of 2 N HCl was added, and the reactions were incubated at 100°C for 10 min to hydrolyze the nucleotide sugars. The samples were desalted on Amberlite MB-3 and chromatographed in Solvent D for 11 h. The paper was cut into 1-cm strips and counted for radioactivity. In addition to radioactivity in the mannose, rhamnose, and 6-deoxytalose regions, a small peak was detected with a mobility between that of rhamnose and 6-deoxytalose. This accounted for 0.5% of the radioactivity in the PL<sup>R</sup>1.3 and control chromatograms and was not detectable in the chromatogram of parent cells due to tailing from the 6-deoxytalose and rhamnose peaks. It appears to represent a trace contaminant in the starting GDP-[<sup>3</sup>H]mannose.

Cell extract	Mannose	Rhamnose	6-Deoxytalose
		cpm	
None	128,120	<120	<120
Parent BW5147	61,295	2,459	4,484
PL <sup>R</sup> 1.3	64,564	<120	<120

ment shown in Fig. 3. Parent or PL<sup>R</sup>1.3 extracts were preincubated without NADPH to allow the formation of intermediates, boiled to inactivate the extracts, and then incubated a second time with fresh extracts as detailed in the figure legend. Panel A contains control samples, demonstrating that this preparation of parental extract produces 0.40 nmol of GDP-fucose in 1 h, after a time lag, and that the PL<sup>R</sup>1.3 extract cannot synthesize GDP-fucose. The data in Panel B show that preincubation with PL<sup>R</sup>1.3 extracts without NADPH does not affect GDP-fucose formation during a second incubation with parental extract. In contrast, as illustrated in Panel C, preincubation with parental extract produces intermediates that PL<sup>R</sup>1.3 extracts can convert to GDP-fucose, and this conversion occurs without the time lag. Because *B* is probably the predominant intermediate (above), the most likely interpretation of these results is that PL<sup>R</sup>1.3 extracts can perform both epimerase and reductase functions. However, we cannot exclude the possibility that one (but not both) of these activities was not inactivated by boiling and thus was present from the preincubations.

When parental cell extracts were mixed with PL<sup>R</sup>1.3 extracts, the conversion of GDP-mannose to GDP-fucose was identical with that observed in control incubations with pa-

rental extract alone (data not shown). This experiment demonstrated that PL<sup>R</sup>1.3 cells do not contain a soluble inhibitor of the 4,6-dehydratase and that the parental cell extracts do not provide compounds (e.g. NADPH) that enable the GDP-mannose to GDP-fucose pathway to function in PL<sup>R</sup>1.3 extracts. The mixing data also suggest that the defective reaction in PL<sup>R</sup>1.3 is the rate-determining step of the conversion pathway.

**Assaying the "Scavenge" Pathway of Fucose Metabolism in PL<sup>R</sup>1.3**—While the predominant pathway for GDP-fucose formation involves the conversion of GDP-mannose to GDP-fucose, a "scavenge" pathway also exists whereby GDP-fucose is formed from free fucose via fucose 1-phosphate (26). We assayed for the presence of this pathway in parent and PL<sup>R</sup>1.3 cells by growing the cells in media containing [<sup>3</sup>H]fucose. Both cell lines incorporated [<sup>3</sup>H]fucose into GDP-[<sup>3</sup>H]fucose, demonstrating that the scavenge pathway is functional in these cells (data not shown). We next subjected the glycoproteins from these [<sup>3</sup>H]fucose-labeled cells to mild base treatment to release O-glycosidically linked oligosaccharides followed by pronase digestion and Bio-Gel P-6 gel filtration chromatography (Fig. 4). The elution profiles of the parental and PL<sup>R</sup>1.3 material were very similar, with most of the radioactivity eluting as glycopeptide species at or near the void volume. Acid hydrolysis of the glycopeptides followed by paper chromatography confirmed that all the label was in fucose.

To determine the magnitude of the scavenge pathway in PL<sup>R</sup>1.3 cells under normal culture conditions, we assayed unlabeled parent and PL<sup>R</sup>1.3 cells for the amount of fucose present in total cellular membrane material. The washed cells

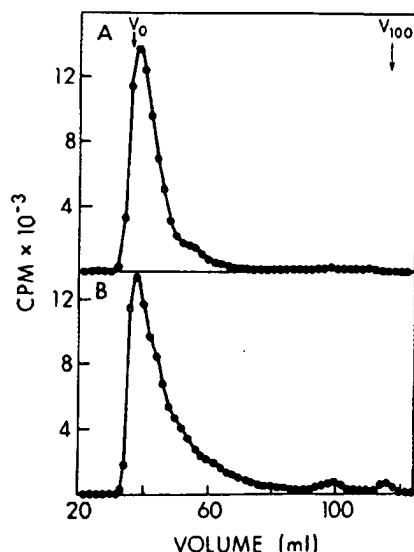


FIG. 4. Parent and PL<sup>R</sup>1.3 cells incorporate radioactivity into glycoprotein upon incubation with [<sup>3</sup>H]fucose. Cells ( $8.3 \times 10^7$  parent and  $5.4 \times 10^7$  PL<sup>R</sup>1.3 at harvesting) were labeled ( $50 \mu\text{Ci}$  of [<sup>3</sup>H]fucose/35 ml, 83 h) harvested, and extracted, as described under "Experimental Procedures." The resulting residual glycoprotein was subjected to mild base treatment ( $0.1 \text{ M NaOH}$ ,  $0.4 \text{ M NaBH}_4$ , under  $\text{N}_2$  in the dark at room temperature for 68 h) followed by neutralization ( $0.1 \text{ M}$  acetic acid) and removal of the borate (addition of methanol followed by evaporation to dryness under reduced pressure, repeated three times). After pronase digestion, the glycopeptides were chromatographed on a column ( $100 \times 1.5 \text{ cm}$ ) of Bio-Gel P-6 using  $0.1 \text{ M NH}_4\text{HCO}_3$  as the column buffer. Each fraction was 2 ml, 10% of which was used for determination of radioactivity. The void volume ( $V_0$ ) was measured with bovine serum albumin, while glucose served as the included volume ( $V_{100}$ ) marker. The elution profile of parent BW5147 glycopeptides is in Panel A and that of PL<sup>R</sup>1.3 glycopeptides is presented in Panel B.

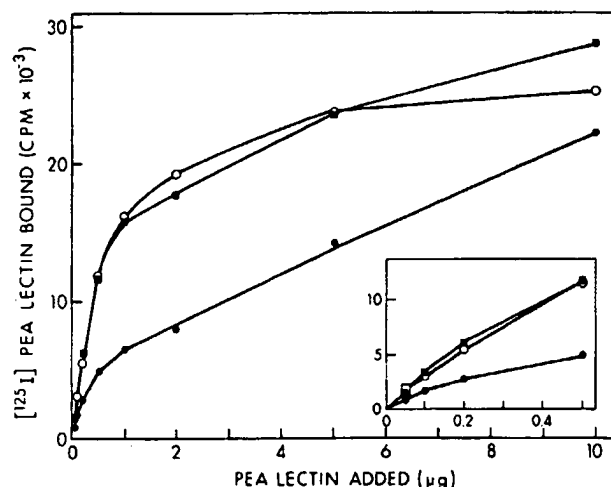


FIG. 5. Specific binding of <sup>125</sup>I-labeled pea lectin to parent BW5147, PL<sup>R</sup>1.3, and PL<sup>R</sup>1.3 cells grown in 10 mM fucose. The PL<sup>R</sup>1.3 cells were grown in complete medium containing 10 mM fucose for 3 days. Binding reactions ( $100 \mu\text{l}$ ) contained  $5 \times 10^5$  cells, <sup>125</sup>I-labeled pea lectin ( $6.7 \times 10^4 \text{ cpm}/\mu\text{g}$ ),  $0.5 \text{ mg}$  of bovine serum albumin, and  $1 \mu\text{mol}$  of  $\text{NaN}_3$  in PBS. Incubations were at  $4^\circ\text{C}$  for 3 h. Separation of cells from free lectin was performed as described (22). Specific binding is defined as binding that is inhibitable by  $0.1 \text{ M}$   $\alpha$ -methyl mannose. Specific binding accounted for greater than 90% of the total pea lectin bound below  $1 \mu\text{g}$  added and for  $>80\%$  of the lectin bound above this concentration.  $\circ$ , parent BW5147;  $\bullet$ , PL<sup>R</sup>1.3;  $\blacksquare$ , PL<sup>R</sup>1.3 grown for 3 days with 10 mM exogenous fucose. The inset shows lectin binding at the low lectin concentrations.

were disrupted by sonication, and the particulate fractions were sedimented. The pellets were then hydrolyzed and the free fucose purified by paper chromatography and assayed by gas-liquid chromatography (see under "Experimental Procedures" for details). Wild type cells contained  $1.52 \text{ nmol}$  of fucose/mg of particulate protein compared to  $0.33 \text{ nmol}$  of fucose/mg of particulate protein in PL<sup>R</sup>1.3 cells.

**Restoration of Pea Lectin Binding to PL<sup>R</sup>1.3 Cells by Fucose Feeding**—We next cultured the cells in medium containing substrate amounts of fucose. These experiments had two purposes, to further demonstrate the functionality of the scavenge pathway and to examine the role of fucose in pea lectin binding. Cells were grown in the presence of 1 mM and 10 mM fucose, since higher concentrations of this sugar (20 to 80 mM) had been shown to cause changes in cell morphology and inhibition of cell growth (27–29). Control experiments demonstrated that 1 mM and 10 mM fucose did not inhibit the growth of parent cells. Fig. 5 shows the specific binding of <sup>125</sup>I-labeled pea lectin to parental and PL<sup>R</sup>1.3 cells and to PL<sup>R</sup>1.3 cells grown in 10 mM fucose. It is evident that fucose feeding restores the ability of PL<sup>R</sup>1.3 cells to bind pea lectin. Scatchard plots of the data demonstrate that PL<sup>R</sup>1.3 cells have approximately one-third the number of high affinity pea lectin-binding sites as either fucose-fed PL<sup>R</sup>1.3 cells or parental cells.

Two other characteristics that distinguish PL<sup>R</sup>1.3 cells from the parent BW5147 line are the mutant's prolonged doubling time (28 h versus 15 h for parent cells) and their more spherical morphology. Neither of these traits was affected by growing the PL<sup>R</sup>1.3 cells in 1 or 10 mM fucose for 6 days.

**In Vitro Fucosyltransferase Assays**—Data presented above indicate that the PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 line's defect may be at the site of fucose transfer from GDP-fucose to glycoprotein. In order to test this hypothesis, we assayed the ability of a detergent extract from each cell line to transfer fucose from GDP-fucose to exogenous fibrinogen glycopeptide and to endogenous acceptors. Table IV contains representative results from one of four experiments. The extract of each mutant line



TABLE IV

## GDP-fucose: glycoprotein fucosyltransferase activity

Washed cells ( $1 \times 10^6$ ) in 8 volumes of 20 mM Tris-maleate, pH 7.4, containing 1% (v/v) Triton X-100 were disrupted by sonication and centrifuged at  $12,000 \times g$  for 10 min. Incubation mixtures (30) of 50  $\mu$ l contained 500  $\mu$ g of the supernatant protein, 40 mM  $MgCl_2$ , 1% (v/v) Triton X-100, 5 mM ATP, 14  $\mu$ M GDP-[ $^{14}C$ ]fucose ( $10^5$  cpm), 20 mM Tris-maleate, pH 7.4, and 2 mM fibrinogen glycopeptide where indicated. After incubating for 5.5 h at 37°C, the entire reaction mixtures were spotted on Whatman No. 3MM paper and developed in Solvent C for 10 h. In this system, the glycopeptide stays at the origin while the GDP-fucose and free fucose migrate with  $R_f$  values of 0.18 and 0.61, respectively. All assays were performed in duplicate, and the maximum variation was 11%.

Source of cell extract	Fib-1 acceptor glycopeptide <sup>a</sup>	Radioactivity at origin cpm
None	+	666
Parent BW5147	-	600
	+	15,466
PL <sup>R</sup> 1.3	-	986
	+	40,488
PHA <sup>R</sup> 1.8 PL <sup>R</sup> 7.2	-	2,467
	+	38,652

<sup>a</sup> Structure shown in footnote 3.

transferred fucose to the exogenous fibrinogen glycopeptide acceptor as well or better than parent cell extracts did. PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 extracts transferred as much or more radioactivity to endogenous acceptors than did either parent or PL<sup>R</sup>1.3. Thus, both PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 and PL<sup>R</sup>1.3 exhibit fucosyltransferase activity in an *in vitro* assay. A simple lack of the fucosyltransferase enzyme can be ruled out as the enzymatic defect in PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2.

$\alpha$ -L-Fucosidase digestion of the fucosylated fibrinogen glycopeptide product released 78 to 95% of the radioactivity as free fucose, demonstrating that the transferred fucose is in  $\alpha$ -linkage. Further characterization of the product was obtained using pea lectin-Sepharose chromatography. The fibrinogen glycopeptide substrate did not adhere to a pea lectin-Sepharose column, while 78 to 94% of the  $^{14}C$ -fucosylated glycopeptide product bound to the column and could be eluted with 10 mM  $\alpha$ -methylglucoside (data not shown). These results indicate that the fucose residue(s) transferred *in vitro* is necessary for pea lectin binding.

## DISCUSSION

The pea lectin-resistant lines analyzed in this study appear to have novel defects in glycoprotein biosynthesis. PL<sup>R</sup>1.3 cells have an absent or greatly diminished ability to convert GDP-mannose to GDP-fucose while the intact PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 cells are unable to transfer fucose from GDP-fucose to glycoprotein acceptors. Although the mechanism of the conversion of GDP-mannose to GDP-fucose has been examined only in bacteria (23), this pathway has been explicitly demonstrated in rabbit tissues (19) and is presumed to be present in the many tissues and cell lines that incorporate radioactivity into fucose after incubation with labeled mannose. The analogous bacterial metabolism of dTDP-D-glucose to dTDP-L-rhamnose and dTDP-6-deoxy-D-talose has been extensively studied and shown to involve at least three physically separable enzymes, a 4,6-oxidoreductase (4,6-dehydratase), a 3,5-epimerase, and a 4-reductase (24, 25, 31). Our results are consistent with the hypothesis that the eukaryotic mouse lymphoma cells use the same pathway as do bacteria and that at least three enzymatic activities are involved in the metabolism of GDP-mannose to GDP-fucose. We have shown that the defect in PL<sup>R</sup>1.3 cells is in GDP-mannose 4,6-dehydratase,

the enzyme that converts GDP-mannose to GDP-4-keto-6-deoxymannose.

The defect in the PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 line is more difficult to discern at the enzymatic level. The intact cells appear to be unable to transfer fucose from GDP-fucose to glycoprotein acceptors in spite of the fact that they can synthesize GDP-fucose and appear to have normal or even increased levels of fucosyltransferase activity as well as the appropriate endogenous acceptors. There are at least four possible explanations consistent with these findings. 1) GDP-fucose may not be transported into the Golgi lumen, resulting in a lack of substrate for the fucosyltransferases. 2) The fucosyltransferases may not be properly located within the cell. This would not have been detected in the *in vitro* assays since detergent was present. 3) A glycosyltransferase may be induced which forms a product that is not an acceptor for the fucosyltransferase. Beyer *et al.* have demonstrated that the synthesis of some oligosaccharides requires a specific order of sugar addition and also that the actions of certain glycosyltransferases are mutually exclusive (32). However, the finding of increased transfer of fucose to endogenous acceptors in the PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2 line is against this explanation. 4) A hyperactive fucosidase could be present. This explanation is improbable because one would expect such an enzyme to be active during the *in vitro* fucosyltransferase assays, resulting in an apparent decrease in fucose transfer to glycopeptide. This was not observed.

A number of other lectin-resistant cell lines fail to carry out specific glycosyltransferase reactions *in vivo*, although they possess the appropriate nucleotide sugars, glycoprotein and glycolipid acceptors, and glycosyltransferases. Clone 13, a wheat germ agglutinin-resistant Chinese hamster ovary cell line, is deficient in galactose, but when assayed *in vitro* it exhibits normal UDP-galactose:glycoprotein galactosyltransferase and UDP-galactose:glucosyl ceramide galactosyltransferase activities (11). Another wheat germ agglutinin-resistant cell line which is deficient in sialic acid contains CMP-sialic acid and *in vitro* sialyltransferase activity (11). Finally, PHA<sup>R</sup>1.8, the immediate parent to PHA<sup>R</sup>1.8 PL<sup>R</sup>7.2, is similar to clone 13. It exhibits galactosyltransferase activity but has reduced glycoprotein galactose, even when grown in galactose to circumvent any possible UDP-glucose:UDP-galactose epimerase deficiency (10).

The finding of diminished glycoprotein fucose content in two separate pea lectin-resistant lines with different enzymatic defects points to a critical role for fucose in the pea lectin cell surface receptor. The restoration of high affinity pea lectin binding when PL<sup>R</sup>1.3 cells are grown in fucose-containing media supports this postulate. In experiments to be published elsewhere, evidence has been obtained that the fucose residue linked to the inner core N-acetylglucosamine of biantennary complex-type glycopeptides is essential for high affinity binding to pea lectin.<sup>4</sup> A previous study of the carbohydrate binding specificity of pea lectin did not examine the role of fucose in the interaction (33).

The fucose residues of oligosaccharides are known to participate in a number of biologic reactions. They serve as blood group determinants (34), as the receptor for macrophage migration inhibitory factor (35-37), and as the determinant recognized by the hepatic fucose (38) and macrophage mannose/fucose receptors (39). Fucose has also been implicated in sperm-egg binding (40), as one of the determinants of the T locus antigens of the mouse (41), and, possibly, in lymphocyte homing (42). A B16 melanoma cell line with a decreased

<sup>4</sup> K. Kornfeld, M. Reitman, and R. Kornfeld, manuscript in preparation.

tendency to metastasize contains glycoproteins that have a decreased sialic acid content and an increased fucose content (12). The finding that pea lectin may be used to select cell lines with defects in fucose metabolism may aid future investigations of the biologic roles of this sugar.

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